Evaluating the Longevity of a Potential Biocontrol for the Rice Blast Disease

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ABSTRACT

Rice Blast, caused by the fungal organism *Magnaporthe oryzae,* is the most important fungal disease of rice and is known to cause yield losses of over $66 billion each year and devastate enough rice to feed 60 million people. With rapidly changing climates and rising global hunger, controlling the Rice Blast disease is increasingly difficult. Currently there are no viable methods to do so as fungicides are expensive, cultural methods ineffective, and the fungus quickly overcomes race specific resistance. This study investigates a prospective biocontrol, *Pseudomonas chlororaphis,* and strives to determine if genomic mutation can induce ability for *M. oryzae* to overcome by the bacterium. *P. chlororaphis* strain EA 105 has shown antagonism to *M. oryzae* in studies performed at the University of Delaware and has the potential to be an effective biocontrol for the Rice Blast disease, but it is important to ask whether this measure will hold up against mutations. *M. oryzae* has an incredibly variable genome and has historically been able to quickly overcome host resistance, so it will likely be able to overcome direct antagonism as well. Based on the tendency of *M. oryzae* to produce diverse mutations, the hypothesis is that genomic mutation will cause *M. oryzae* to overcome antagonism by the EA 105 strain of *P. chlororaphis*. To test this hypothesis KJ201 and Guy11 strains of *M. oryzae* were transformed using a plasmid containing genes encoding for both Green Fluorescent Protein and Hygromycin resistance. The random insertion transformants were subsequently exposed to *P. chlororaphis* EA 105. An average of 2% of Guy 11 transformants and 9% of KJ201 transformants were able to successfully overcome *P. chlororaphis* and the hypothesis was confirmed. Further molecular studies are currently being conducted to assess how the gain of function mutation took place to learn more about how *M.oryzae* responds to biocontrol.

INTRODUCTION

In the year 2050 the Earth's population will reach an estimated 9 billion people, yet this incredible milestone hits amidst a rapidly changing climate and increasingly prevalent plant disease pressures threatening critical crops. Due to the lack of global monitoring systems and record keeping, it is unclear what percentage of yield is lost every year to diseases, but the impact of plant diseases is incontestable. Plant diseases are only going to rise as many climates worldwide become more conducive to fungal and bacterial growth and less conducive to plant growth. A fungal organism called *Magnaporthe oryzae* is the causal agent of the Rice Blast Disease that is currently ravaging rice yields. This important crop provides 21% of the world's caloric intake, (Awika 2011) but to this date, there are no viable methods of controlling or containing the fungus. Resistance has not been long lived; many fungicides are not cost effective or available in all the areas rice is grown. In addition, cultural methods alone have not proved effective enough. However, recently strain EA105 of *Pseudomonas chlororaphis* native to rice rhizomes has been isolated and shows significant antagonism to *M. oryzae*. Potential biocontrol is incredibly exciting, but the variable nature of *M.oryzae's* genome calls in the question of longevity. This study will determine if bio control by EA 105 can be a viable component of a solution to one of the most devastating fungal diseases of the global food supply.

State of Hunger and Food Security Today

According to the Food and Agriculture Organization, (FAO), undernourishment has been rising since 2014 from 775.4 million people to an estimated 815 million in 2016. Global hunger now affects 11% of the world's population. After over 10 years of decline in world hunger, regions such as South Eastern and Western Asia as well as sub-Saharan Africa are facing significant food security issues (FAO et al. 2017). Sub-Saharan Africa, in particular, is in a precocious position due to severe droughts, floods, and other climactic changes. This vulnerability is only exacerbated by conflict, limiting the amount of production that can be achieved. A shocking statistic by the Food Insecurity Experience Scale showed that 26% of the sub-Saharan African population ages 15 and above experienced severe food insecurity in the year 2014/2015. This number is 18.7% higher than the world average at 7.5% (Amegbeto 2017). With such disparate statistics, it is clear that to conquer global food insecurity it is critical to address developing countries first.

Background on Rice

Rice is a staple for over 2.4 billion people in the continent of Asia alone, (Rice in Asia). Yet rice is a global crop, eaten across regions and continent and provides 21% of the world's caloric intake, (Awika 2011). In developing countries grain crops contribute up to 80% of caloric intake, with rice being the most important. More recently rice consumption has been falling in Asia due to increases in meat consumption, but rice consumption outside of Asia is rising rapidly. The demand for rice in sub-Saharan Africa in particular is particularly significant, increasing by more than 50% in the past twenty years alone, (Mohanty). While production is increasing, the demand for rice in sub-Saharan Africa still outweighs supply, forcing crucial capital to be spent on the importation of the crop. Increasing rice yield will be crucial to improving food security in sub-Saharan Africa and therefore food security worldwide (Awika 2011).

The Threat of Rice Blast

With a rapidly increasing population and rise in rice consumption worldwide, the demand for quality high yielding rice is staggering. Yet this increasing in demand comes at a time where cultivatable land and water for irrigation are decreasing, so rice must be grown in a much more intensified manner. Generally, this results in higher nitrogen levels from increased fertilization and reduced water, creating an environment highly conducive to many plant pathogens, including the Rice Blast disease. Rice Blast is present globally in all areas where rice is grown and has the ability to devastate entire fields, averaging 60-100% of yield loss. Rice Blast costs over 60 billion a year and destroys enough rice to feed 60 million people, (Pennisi 2010).

Control Through Host Resistance

For most plant diseases, the most effective management strategy is host resistance. Many important plant diseases are managed through single gene race specific resistance where corresponding genes in the host plant and pathogen enable the host plant to recognize the invader and trigger an immune response, therefore conferring resistance. Over 80 single-locus, race specific resistance genes have been found for *M.oryzae*, but when deployed, the resistance has been short lived and overcome in 2-4 years (Ballini et al. 2008). *M. oryzae* is a highly variable fungus and develops new races quickly (Valent and Chumley 1994). Solitary resistance genes are

now considered ineffective in controlling the disease and many researchers now look to stack resistance genes or opt for quantitative resistance (Zhou, Jia, Singh, Correll, & Lee 2007).

Potential for Biocontrol

As single gene resistance seems more and more futile, scientists are looking for other control options for Rice Blast and other plant pathogens. As interest in phytobiomes increases, an emerging management strategy is biocontrol, or using another living organism to control the pathogen. Types of biocontrol fall into two broad categories – antagonism and host induced protection. Antagonism refers to when the biocontrol directly opposes or inhibits growth or fitness of the target pathogen. This can be through antibiosis, when the biocontrol secretes a metabolic product that inhibits the pathogen, competition for resources/habitat, hyperparasitism, or predation. While antagonistic processes concern solely the relationship between the biocontrol and the pathogen, induced protection mainly concerns the relations between the biocontrol and the host plant itself. Induced protection can be achieved through induced resistance, inhibitors/competition, and hypovirulence. The most intriguing of these is induced resistance where the control is used to "prime" the plant it can prepare the plant for infection and reduce total disease incidence (Cate 1990; Hoy and Herzog 1985)

Biocontrol is an increasingly prevalent possibility for controlling many plant pathogens (Adams 1990). Although studies of plant microbiomes are relatively new, there is hope that biocontrol has the potential to be a more sustainable and cost-effective way to manage diseases. Questions regarding dissemination and execution must still be addressed, but researchers are already looking for candidates to manage some of the world's most aggressive plant pathogens. In 2014, researchers at the University of Delaware worked to isolate bacteria native to the rice rhizosphere and lead a study to evaluate their potential as biocontrol for the Rice Blast disease. Of the 11 bacteria that were studied, one *Pseudomonas chlororaphis* strain named EA 105 stood out in its ability to directly inhibit growth of *M. oryzae* by 75%, reduce appressoria (fungal structures critical for infection) formation by 90%, and trigger induced systemic resistance in rice (Spence et al. 2014).

Pseudomonas strains have long been known for demonstrating fungistatic activity, and the University of Delaware findings are promising, but the idea of direct antagonism brings an important question. If *M.oryzae* is so easily able to overcome host resistance, then is it possible it could very quickly overcome direct antagonism by *P. chlororaphis* EA105? Genetic studies of *M. oryzae* populations have shown that while the overall rate of mutation is relatively standard, specific regions of the *Magnaporthe* genome have significant rates of mutations. These regions tend to be related to host specificity and even cultivar specificity, and thus are required for the fitness of the species. This evolutionary pressure is likely a reason for these highly variable pieces of genetic information (Valent and Chumley 1994). There is extensive research on the interactions between rice resistance genes and *M. oryzae* avirulence genes, but little has been done to study interactions between *M. oryzae* and other microbial organisms and not much is known on what genes influences these relationships. For these reasons it is likely that when continually exposed to selection pressure by *P. chlororaphis* EA105, *M. oryzae* may evolve resistance to the bacterium and the biocontrol would no longer be effective.

By inducing mutations in *M.oryzae* through a random insertion and consequently exposing the mutants to *P. chlororaphis* EA105, this study strove to determine if *P. chlororaphis* EA105 can be an effective long term biocontrol against the Rice Blast disease.

OBJECTIVES AND HYPOTHESIS

Although the molecular basis of antagonism by *P. chlororaphis* is currently unknown, it is unlikely that it functions as a single-gene like host resistance does. This means that antagonism should be harder to overcome, but *M. oryzae* is nonetheless a highly variable organism. For EA 105 to be a viable long-term strategy to alleviate the pressure of Rice Blast, it must withstand mutations by *M. oryzae.* Based on the plasticity of *M. oryzae*'s genome, the hypothesis is that induced mutations of the *M. oryzae* genome will result in an ability to overcome antagonism by the EA 105 strain of *P. chlororaphis.*

MATERIALS AND METHODS

P.chlororaphis isolate EA 105 was generously provided by Dr. Harsh Bias (University of Delaware) and frozen in glycerol for storage. *M.oryzae* isolates Guy 11 and KJ201 were used for transformations and maintained on oatmeal agar in a 27°C incubator throughout the experiment.

Plasmid Preparation BHtz-sGFP plasmid containing *Escheria coli* were grown out on LB agar from stored glycerol stock. Prior to plasmid preparation, 5mL of LB broth was inoculated with an *E.coli* colony in 15mL falcon tubes and grown overnight shaking at 37°C. A basic DNA extraction was used to extract and prepare the plasmid for transformation.

Fungal Transformation Spores of actively growing cultures of *M. oryzae* isolates Guy 11 and KJ201 were used to inoculate 200mL of CM broth (1.5g yeast extract, 1.5g casamino acids, and 2.0g sucrose in 200mL dd H_2O) in 1L flasks with 100ug/mL ampicillin to prevent contamination. The cultures were grown shaking at room temperature for 2-4 days until sufficient mycelia had grown. The mycelium was harvested through autoclaved miracloth and placed in 50mL falcon tubes before washing three times with 20% sucrose solution. Lysing enzymes from *Trichoderma harzianum* were prepared in the 20% sucrose solution (approximately 0.23g in 5mL solution) and added to the mycelia through a 0.22um syringe filter to sterilize. The mycelia/enzyme solution was volumed up to 20mL with the 20% sucrose solution and left on a shaking platform for 3 hours.

A drop of solution was added to a glass slide and examined under a microscope for protoplasts. The solution was then filtered through several layers of autoclaved miracloth and centrifuged to collect the protoplasts. The supernatant was discarded, and the protoplasts resuspended with 5ml 1XSTC (20% sucrose, 50mM TrisHCl pH 8.0, and 50mM $CaCl_2$). 200uL protoplast solution was transferred to a 15mL falcon tube. 5uL prepared plasmid DNA was mixed with equal volume 1xSTC for appropriate osmocompatibility. 5uL of the DNA/STC mixture was added to the protoplasts. After 10min of incubation at RT, 1mL PTC (1:1 mixture of 2xPEG (4g polyethylene glycol in 5mL H_2O) and 2xSTC) was added by carefully dropping over the protoplast mixture. The solution was incubated at RT for 20min to allow the protoplasts to come together. 3mL TB3 broth (0.3% yeast extract, 0.3% casamino acids, 1.0% glucose, and 20% sucrose) was added and the solution left to mix gently on a shaking platform for 6 hours. 12 plates were prepared for

each sample (KJ201 and Guy 11) with 500uL protoplast mixture in 20mL TB3 agar (0.3% yeast extract, 0.3% casamino acids, 1.0% glucose, 20% sucrose, and 0.8% agar) with 150ug/mL Hygromycin. The plates were wrapped in foil and placed in an incubator set to 27°C to allow the protoplasts to grow through the agar. Once hyphal growth was visible on the surface of the agar, colonies were selected randomly and viewed under a fluorescent scope to confirm the transformation was successful.

Challenging with *P. chlororaphis* Once the fungal colonies were visibly grown through, (1-2 weeks), they were exposed to P.*chlororaphis* to select for resistant colonies. 20mL LB broth was inoculated with *P.chloraraphis* EA105 and grown shaking overnight at 37°C and diluted to O.D. 600 = 0.075A. 500uL of the bacterial solution was pipetted over the plates before a thin layer of LB agar was poured over top. The plates were sealed with parafilm, covered with foil, and left to grow in an incubator set to 25°C for 5 days. Transformed fungal colonies were counted using Colony Count software before and after exposure to the bacteria.

Transformed colonies that had successfully grown through the bacteria and LB agar were randomly selected and subcultured onto oatmeal agar. After a week of growth, they were checked under the fluorescent scope to once again confirm successful transformation. To evaluate the significance of their ability to withstand *P.chlororaphis* EA105 they were subjected to a series of tests from the original University of Delaware study (Spence et al. 2014).

Direct antagonism test Transformed isolates were tested, along with wild type Guy 11 and KJ201 for ability to withstand direct antagonism by *P.chlororaphis*. The tests are identical to those performed in the University of Delaware paper and the same procedures were followed (Spence et al. 2014). CM (10 g sucrose, 6 g yeast extract, 6 g casamino acids, 1 ml *Aspergillus nidulans* trace elements, and 15 g agar, for 1L media) agar was prepared and poured into sterile KDFAL petri dishes. Squares of each isolate were plated 4 cm away from 5ul of bacteria (O.D. 600 = 0.075). The initial trial (2.25) tested 14 isolates against the KJ201 and Guy 11 wild types. One biological replicate of each mutant was used and three biological replicates of the wild types were used. Inhibition was calculated against the wild type controls. In the following trials, (3.17, 3.25, 3.29 and 4.8), two biological replicates were performed for each isolate as well as two control plates per isolate with only *M. oryzae.* Two control plates of *P.chlororaphis* EA105 were also prepared, with 5ul of bacteria only. plates were sealed with parafilm, covered in foil, and set in a 25°C incubator for 5 days. Photographs were taken and ImageJ was used to measure the diameter of mycelial growth. Percentage inhibition was calculated against control plates using averages for each isolate as well as against the wild type using the formula ([C-T) x 100]/C. C = fungal diameter in control plate or wild type and T = fungal diameter in the treated plates.

Appressoria test Transformed isolates were tested along with wild type Guy 11 and KJ201 to determine if there was any change in germination or appressoria inhibition. This test is based off those performed in the University of Delaware paper and the same procedures were followed (Spence et al. 2014). *M. oryzae* isolates were grown on oatmeal agar for 10 days in an incubator at 27. Spores were collected using sterile H2O and harvested through autoclaved miracloth. Spores were counted using a hemocytometer and the spore solutions diluted to 2.0x10^5 spores/mL. *P. chlororaphis* was grown overnight and diluted to O.D.600 = 0.04. 1:1 spores to

bacteria solutions were made for final concentration of 1.0x10^5 spores/mL and O.D.600 = 0.02. Control solutions used sterile H2O instead of bacteria. Coverslips were used as hydrophobic surfaces to allow appressoria formation. Three plastic coverslips were placed in sterilized glass petri dishes containing a sterilized filter disc. 50uL drop of spore mixtures were pipetted onto each coverslip and the filter disc moistened with sterile H2O. The petri dishes were kept in the dark and photographs taken at 3 hours post treatment for germination and 24 hours post treatment for appressoria formation using a Carl Zeiss Axioscope pro. 5 images were taken per coverslip for a total of 15 images per treatment. Percent germination was determined by dividing the number of germinated spores by the number of total spores per treatment. Percent germination and appressoria formation was calculated by averaging the total number of spores per treatment and dividing the germinated spores by the total spores and the number of spores with appressoria by the number of spores germinated. Inhibition was calculated using the formula [(C-T)/C] where C=untreated and T=treated.

Isolation of genomic DNA and inverse PCR Transformed isolates were grown in 125mL CM broth with 100ug/mL ampicillin shaking at room temperature for 48 hours before mycelia was harvested and DNA extracted using CTAB buffer (2% CTAB, 100mM Tris HCl, 10mM EDTA, and 0.7M NaCl) to yield high quality, high concentration DNA for inverse PCR. >500ng genomic DNA was digested with MSPI enzyme for 12-18 hours at 37°C. The digest was extracted with 1:1 chloroform:isoamyl alcohol and then precipitated with ethanol and 3M NaAOc. The digest was then ligated with T4 ligase enzyme for 12-18 hours at 15°C to form a plasmid with known regions. The ligate was again extracted with 1:1 phenol:chloroform and then precipitated with ethanol and 3M NaAOc. 10ul of the ligated sample was used in a PCR reaction with primers selected based on the BHtz-sGFP plasmid. Subsequent rounds of PCR were used to narrow down the amplified genes before being sent out for sequencing.

RESULTS

A total 20 Guy11 colonies and 620 KJ201 colonies grew through *P.chlororaphis* EA105, representing of 2% of the Guy 11 background mutants and 9% of KJ201 background mutants. 180 of these mutants were picked and used for further studies. All 180 mutants were successfully transformed and able to withstand Hygromycin as well as produce green fluorescent protein under fluorescent light.

Plate	Colonies before EA105	Colonies after EA105	Percentage Grown
Guy11-1	285.5	0	0%
Guy11-2	185.5	2	1%
Guy11-3	286.5	0	0%
Guy11-4	93.5	4	4%
Guy11-5	319.5	14	4%
Guy11-6	386	0	0%
TOTAL	1556.5	20	
		Average percent Guy 11	**2%**
KJ201-A1	143.5	0	0%
KJ201-A2	215	8	4%
KJ201-A3	122.5	0	0%
KJ201-A6	156.5	0	0%
KJ201-A7	232.5	0	0%
KJ201-A8	165.5	14	8%
KJ201-B2	300	61	20%
KJ201-B3	394	47.5	12%
KJ201-B5	813	113.5	14%
KJ201-B6	837	123	15%
KJ201-B7	492	0	0%
KJ201-B9	507	94.5	19%
KJ201-C3	711.5	64	9%
KJ201-C4	305.5	7.5	2%
KJ201-C6	219.5	2	1%
KJ201-C7	163.5	73	45%
KJ201-C8	377.5	12	3%
TOTAL	6156	620	
		Average percent KJ201	**9%**

Table 1: Fungal colonies before and after exposure to *P.chlororaphis* EA105

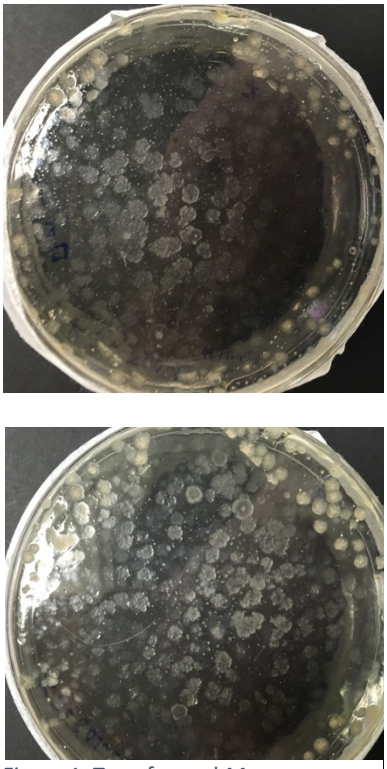


Figure 1: Transformed *M. oryzae colonies* before exposure to *P. chlororaphis* EA105

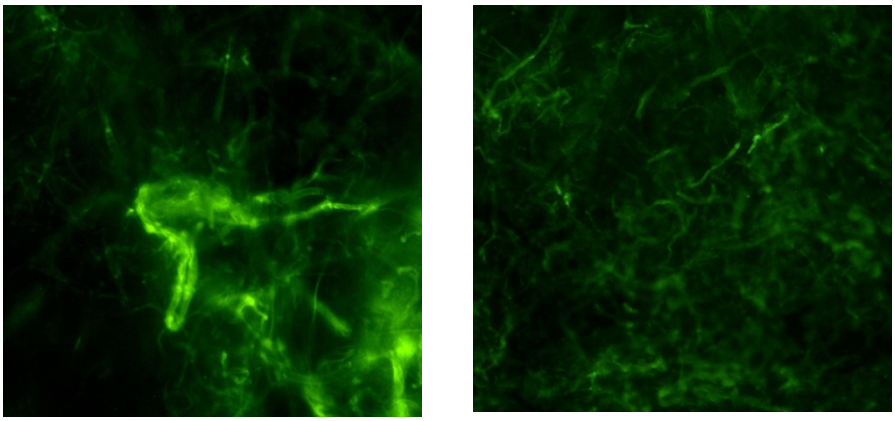
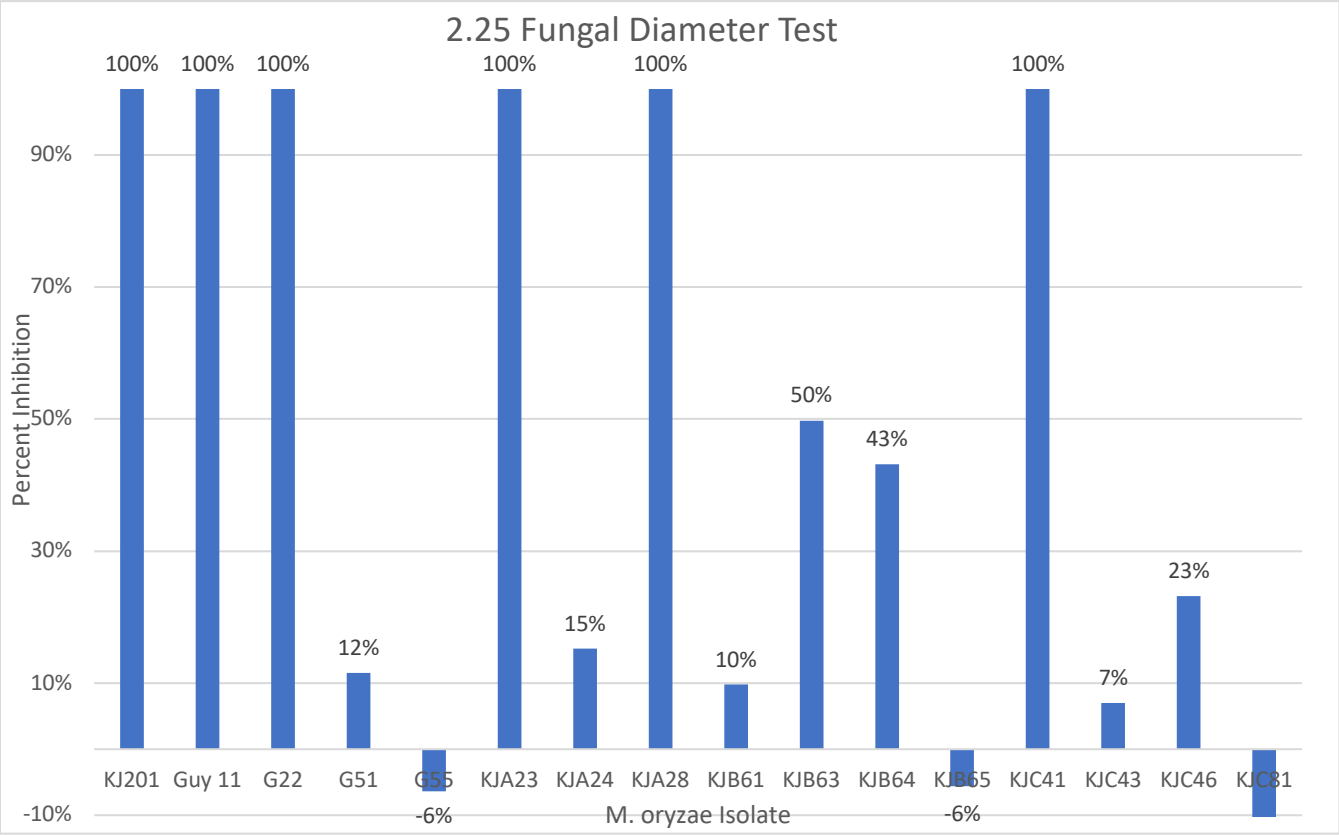


Figure 2: Transformed *M. oryzae* fluorescing green

Direct Antagonism Test

2.25 Fungal Diameter Assay

The first fungal diameter assay was conducted on February 25th, 2019. Only one biological replicate was used per isolate and inhibition was calculated against wild type controls. KJ201 and Guy 11 wild types were inhibited 100% along with G22, KJA23, KJA28 and KJC41. The rest of the transformed *M. oryzae* isolates showed significant reduction in inhibition.



Graph 1: February 25th fundal diameter test.

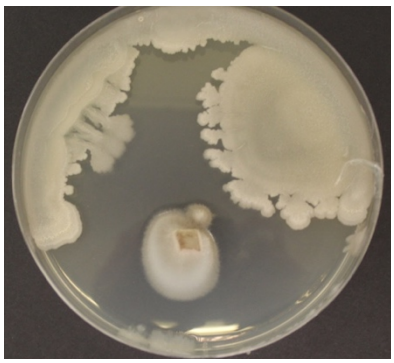


Figure 4: Guy 11 Wild Type in 2.25 antagonism test

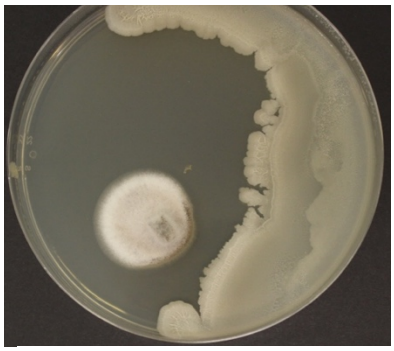


Figure 4: KJ201 mutant A24 in 2.25 antagonism test

Note: Analysis of variance test could not be used for the 2.25 initial trial as only one biological replicate was used.

3.17 Fungal Diameter Assay

In the second trial, evaluated on March 17th, Guy 11-22 and Guy11 -55, (mutants with Guy 11 backgrounds), continued to show reduced inhibition by *P. chlororaphis* EA105. KJ201 mutant KJA21 showed higher inhibition than its wild type KJ201 while the rest of the KJ mutants, KJA27, KJB93 and KJC35 showed reduced inhibition.

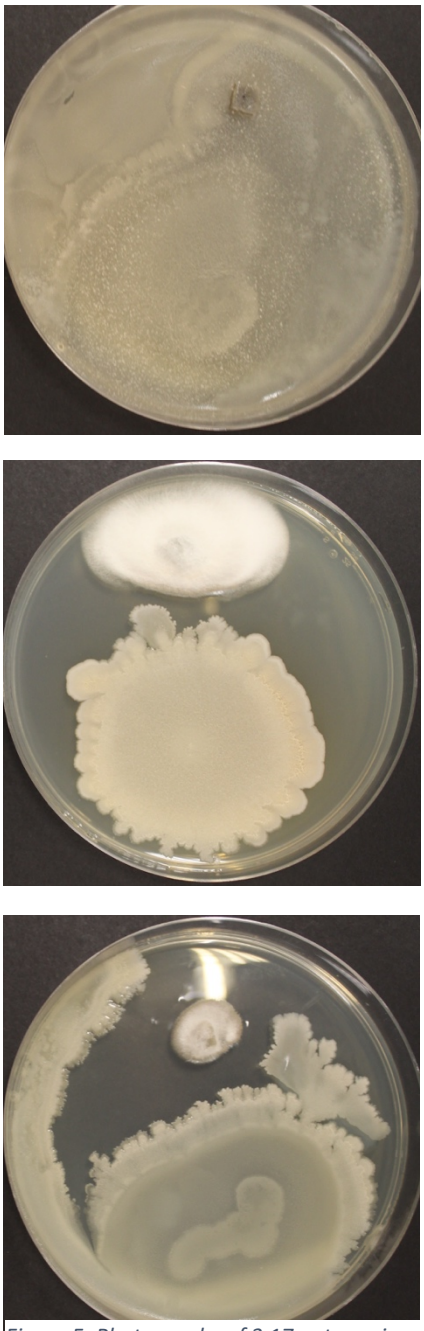
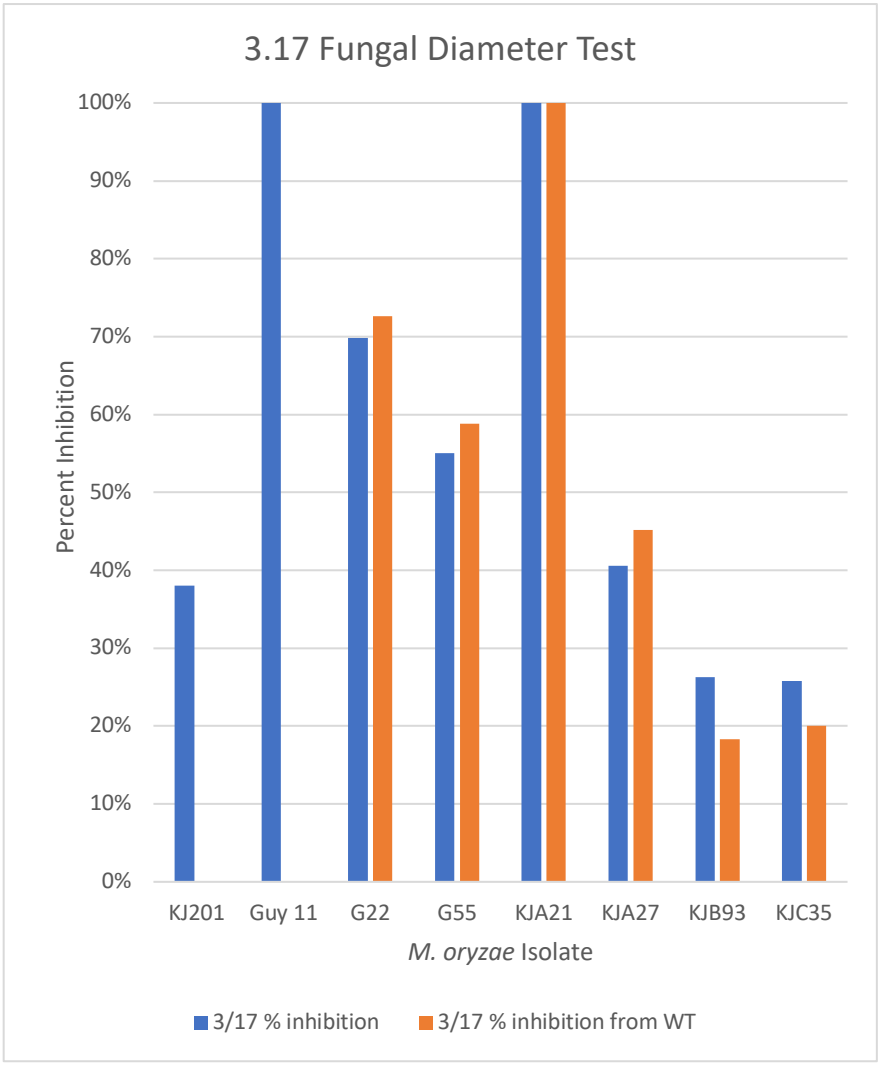


Figure 5: Photographs of 3.17 antagonism trial. Top: Guy 11 Wild type, Middle: Guy 11 mutant 22, Bottomt: Guy 11 mutant 55.



Graph 2: March 17th fungal diameter test

Table 2: Analysis of Variance for 3.17 fungal diameter assay

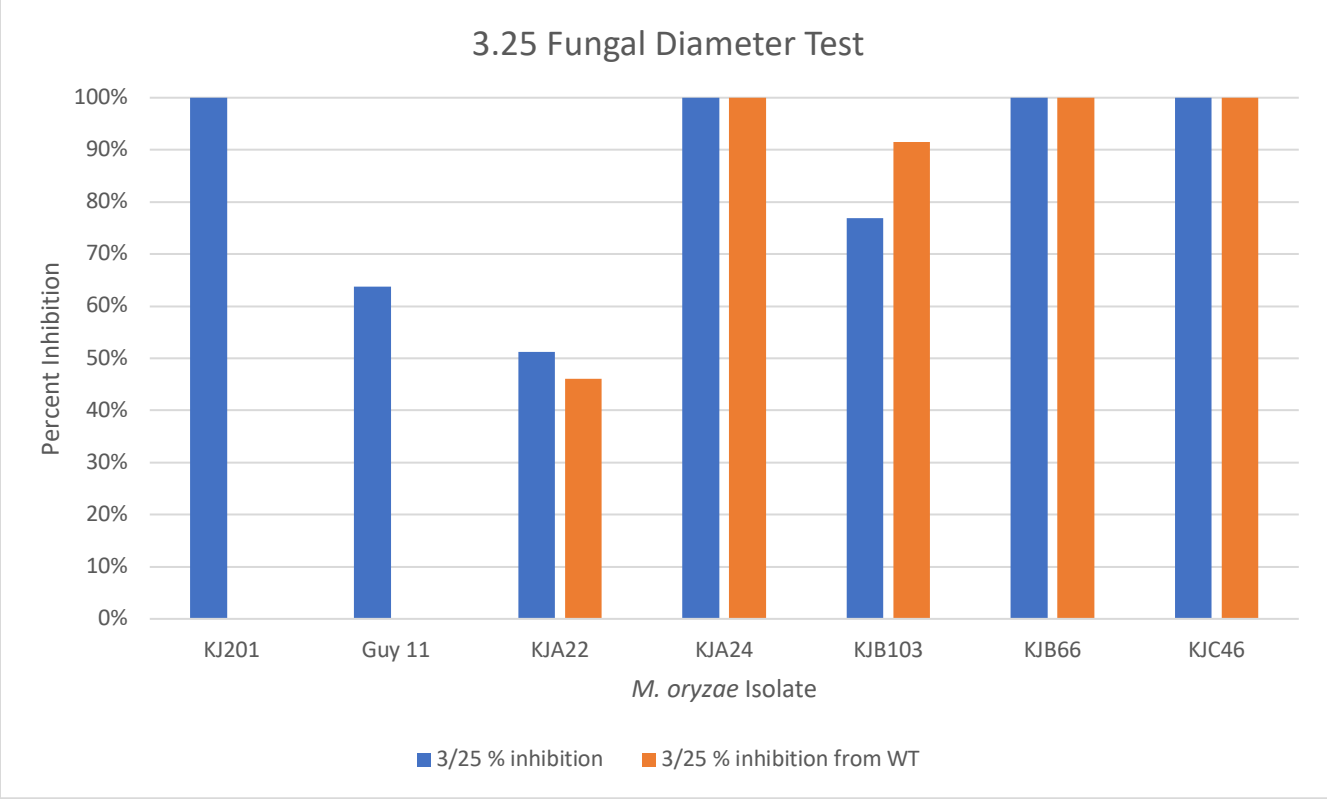
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9612.66999	15	640.844666	5.76033443	0.00204774	2.61685123
Within Groups	1335.01554	12	111.251295			
Total	10947.6855	27				

An analysis of variance of the individual data showed a significant difference between groups. The F value – 5.760 is well above the F crit – 2.617, indicating that there is significant variance between trials.

3. 25 Fungal Diameter Assay

The third trial, evaluated on March 25th, KJ201 mutant KJA24 showed equal inhibition to the KJ201 wild type, a reverse of an earlier trial (see Graph 1). KJB66 and KJC46 also showed equal inhibition to wild type KJ201, while mutants KJA22, KJB103 and KJB75 showed reduced inhibition.



Graph 3: March 25th fungal diameter test

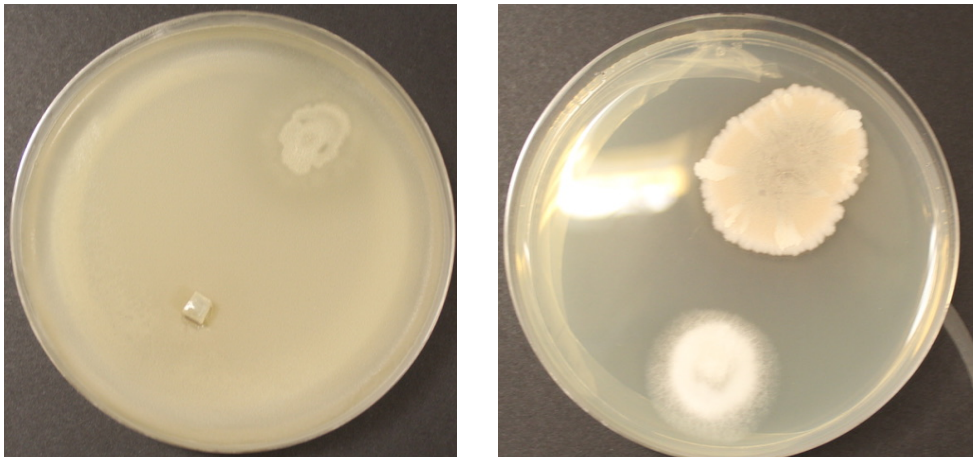


Figure 6: 3.25 antagonism tests. Left: KJA24, Right: KJA22

Table 3: Analysis of variance for 3.25 fungal diameter assay

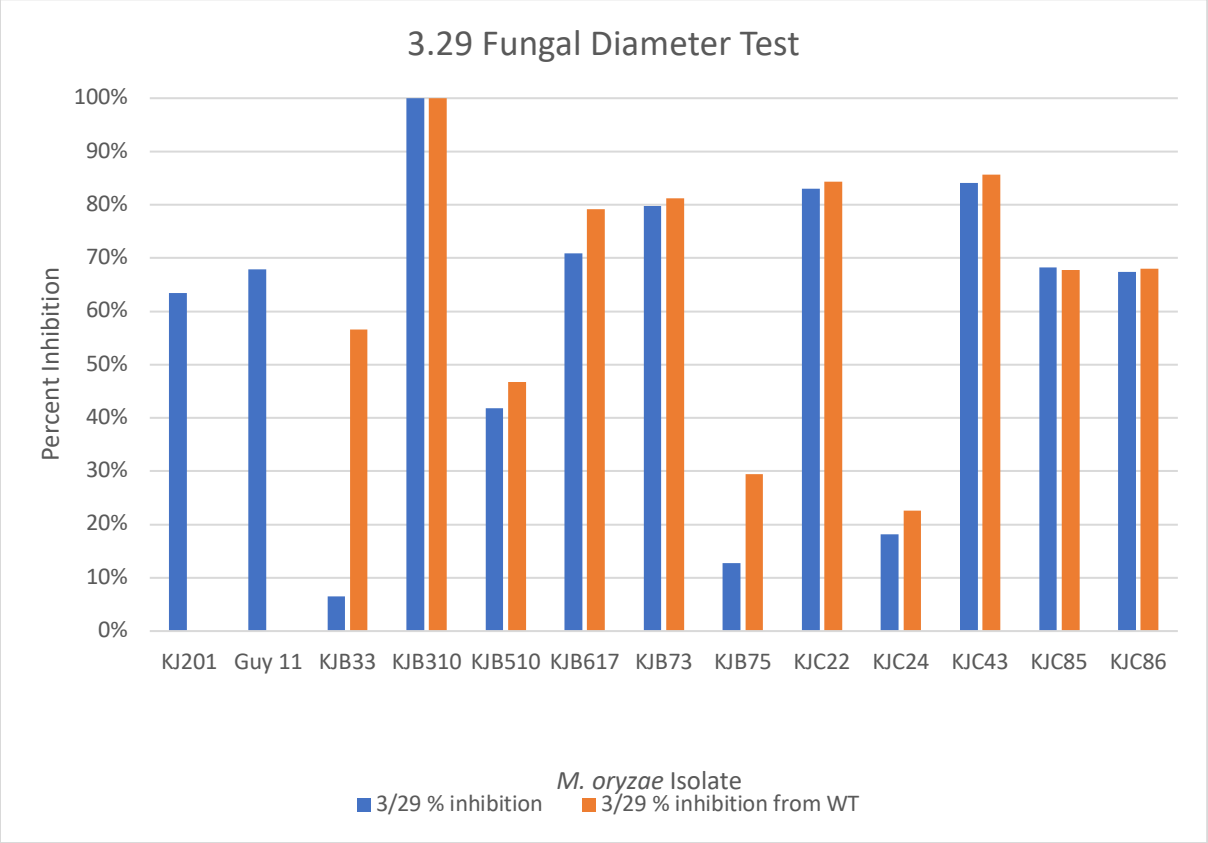
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6401.27906	9	711.253229	6.18346872	0.0043692	3.02038295
Within Groups	1150.24958	10	115.024958			
Total	7551.52864	19				

An analysis of variance of the individual data showed a significant difference between groups. A low P-value and F value – 6.183 well above the F crit – 3.02 indicates that there is significant variance between trials.

3.29 Fungal Diameter Assay

In the fourth trial, evaluated on March 29th, the majority of KJ201 mutants showed higher inhibition than wild type KJ201. This included KJB310, KJB617, KJB73, KJC22, KJC43, KJC85 and KJC86. KJC24, KJB33, KJB510 and KJB75 exhibited low inhibition.



Graph 4: March 29th fungal diameter test

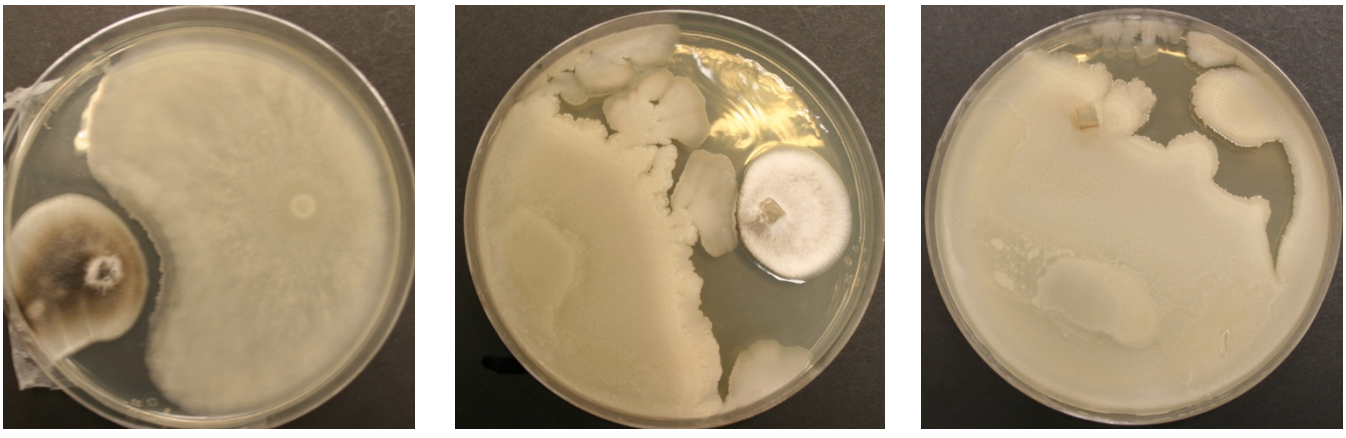


Figure 7: 3.29 direct antagonism test. Left: KJC24, Middle: KJC86, Right: KJ201

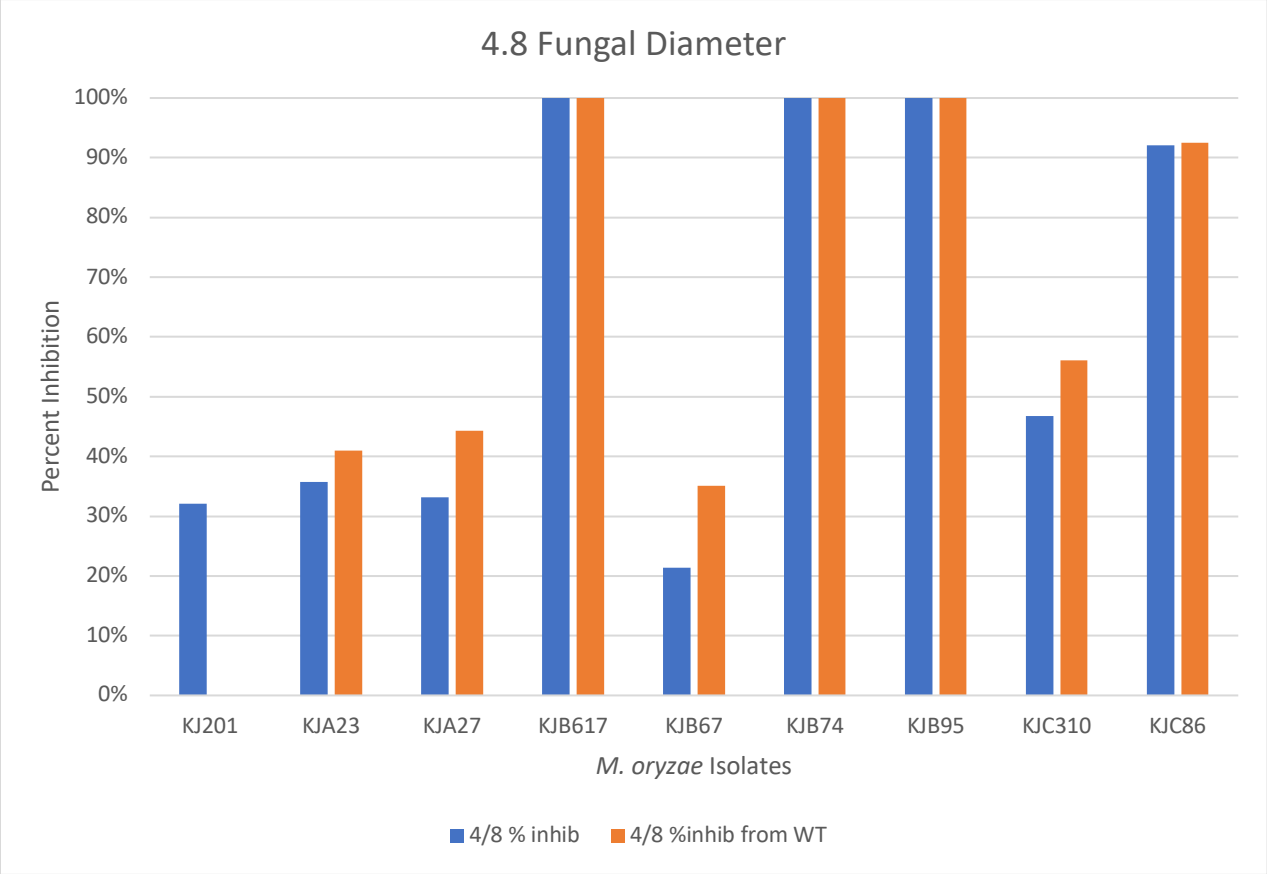
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14582.6845	29	502.85119	4.15944242	0.00022319	1.90740483
Within Groups	3143.24124	26	120.893894			
Total	17725.9257	55				

An analysis of variance of the individual data showed a significant difference between groups. A low p-value and F value – 4.160 well above the F crit – 1.907 indicates that there is significant variance between trials.

4.8 Fungal Diameter Assay

The final trial, evaluated on April 8th, the mycelial growth was only inhibited was only inhibited by 32% in the wild type KJ201. KJB617 continued to show high inhibition (see graph 4) along with KJB74 and KJB95. KJA23, KJA27 and KJB67 showed low inhibition, even if inhibition in KJA23 and KJA27 were slightly higher than KJ201.



Graph 5: 4.8 fungal diameter test.

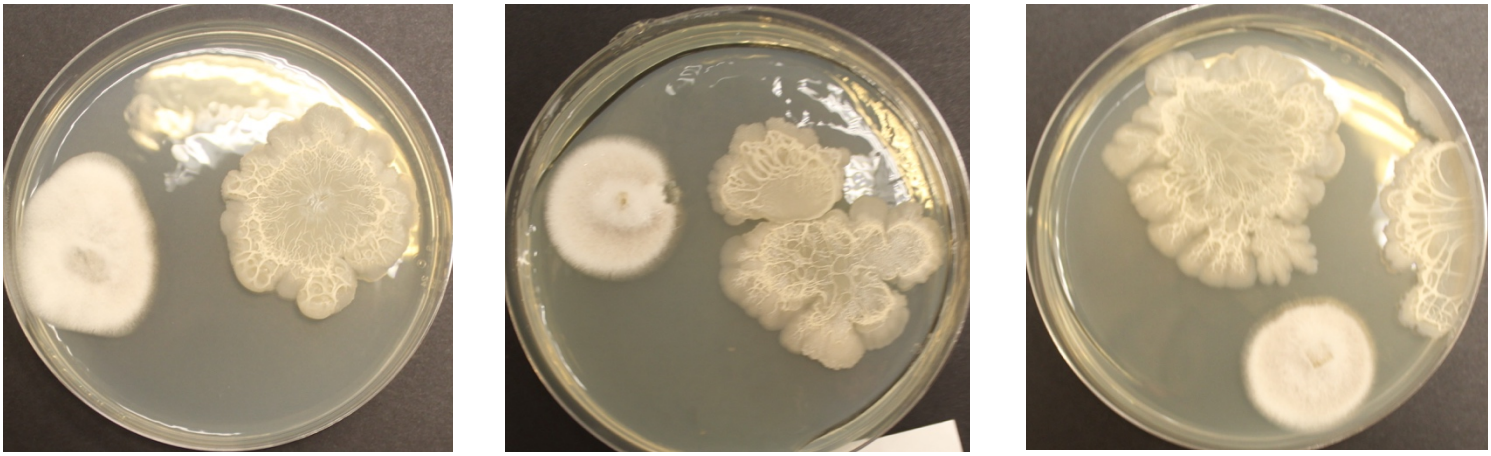


Figure 8: 4.8 direct antagonism test. Left: KJB67, Middle: KJA27, Right: KJ201

Table 4: Analysis of variance for 4.8 fungal diameter assay

ANOVA

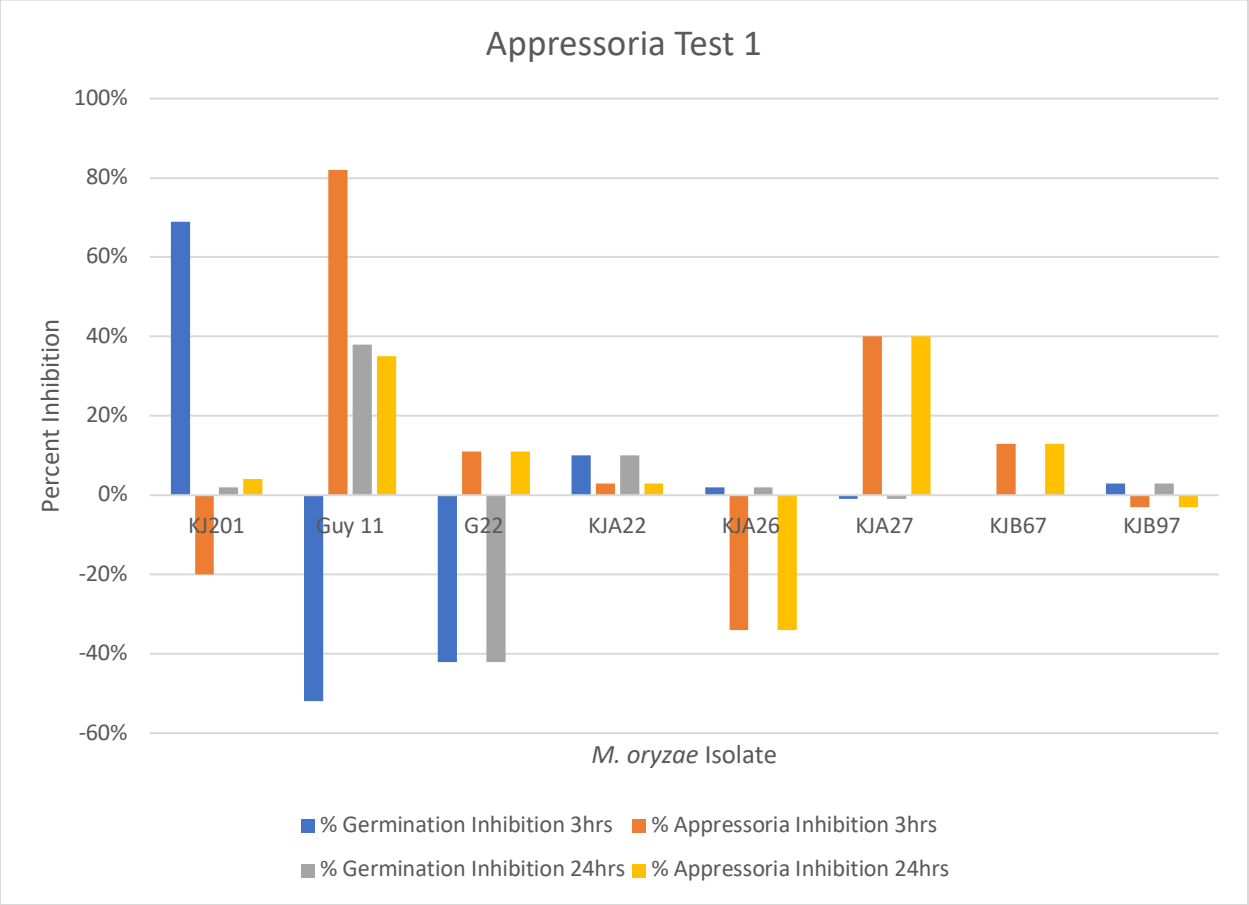
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8109.91147	15	540.660764	30.0330488	2.7554E-09	2.30769267
Within Groups	306.037295	17	18.0021938			
Total	8415.94876	32				

An analysis of variance of the individual data showed a significant difference between groups. A very low p-value and F value – 30.033 far above the F crit – 2.308 indicate that there is very significant variance between trials.

Appressoria Test

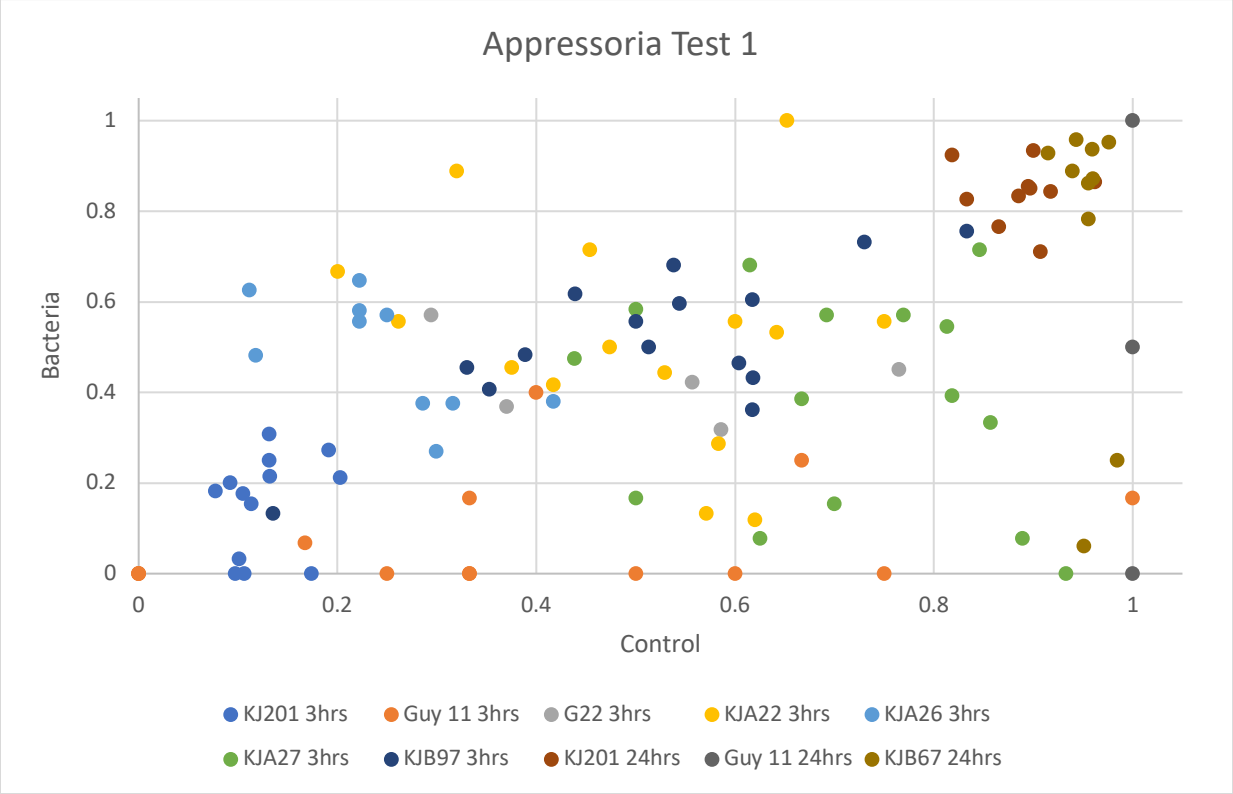
Appressoria Test 1

Germination inhibition was significantly higher in the wild type KJ201at 3 hours but was the appressoria inhibition was actually negative at 3 hours. Nonetheless the appressoria inhibition was still lower at 24 hours in all KJ mutants except KJA27 and KJB67.



Graph 6: Initial appressoria test showing relation between germination and appressoria inhibition at 3 and 24 hours

Appressoria ratios were compared with bacterial treated trials on the Y-axis and the control on the X-axis at both 3 hours and 24 hours. The scatter plot showed that isolates were generally clustered with some variance. Interestingly enough KJ201 clustered close to (0,0) at 3 hours and (1,1) at 24 hours (see graph 7). Analysis of variance tests were run for appressoria ratios, with control and bacterial treatments as separate groups, at both 3 and 24 hours. Both time periods showed significant variance. At 3 hours there was a very low p-value of 1.66E-19 and low F crit – 1.777 compared to an F-value of 12.904. At 24 hours there was a low p-value of 0.00159 and relatively low crit – 2.89 compared to the F-value of 4.548 (see table 5)



Graph 7: Appressoria ratios from trial 1

Table 5: Analysis of variance test for trial 1 appressoria ratios.

ANOVA Appressoria Trial 1 at 3 hours

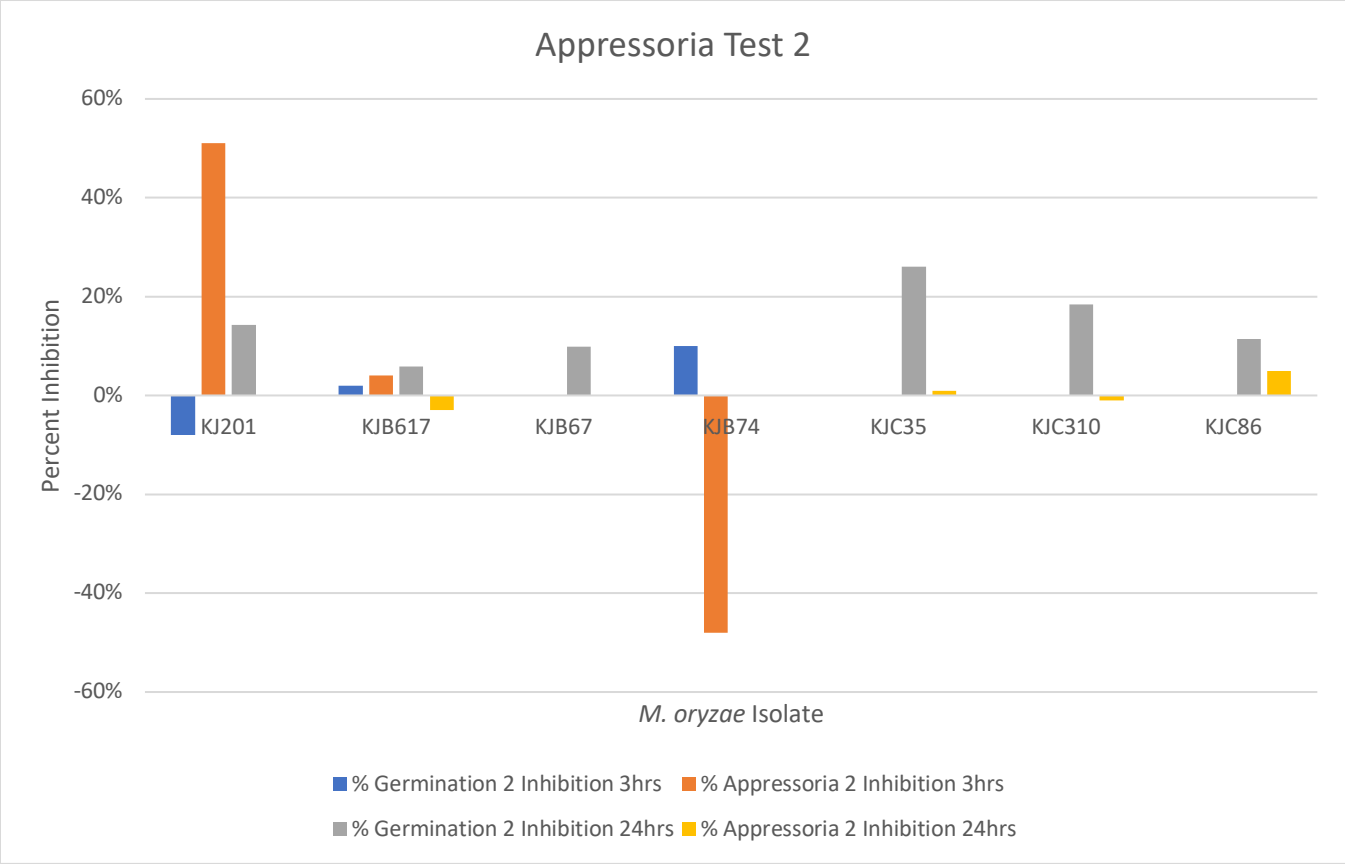
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.6549716	13	0.43499782	12.9039341	1.6662E-19	1.77777177
Within Groups	5.76449215	171	0.03371048			
Total	11.4194638	184				

ANOVA Appressoria Trial 1 at 24 hours

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.7058261	5	0.14116522	4.54827422	0.00159338	2.38944376
Within Groups	1.64496604	53	0.0310371			
Total	2.35079214	58				

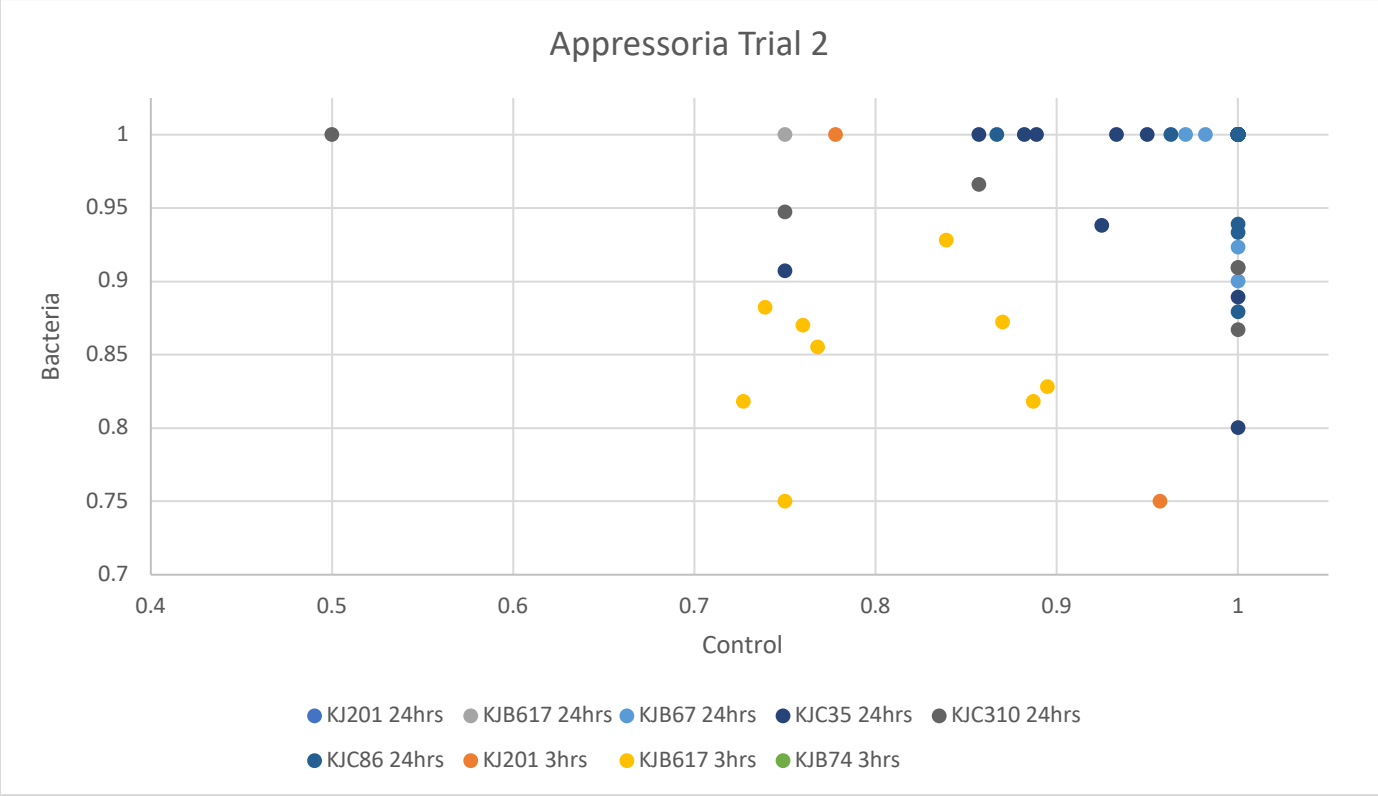
Appressoria Trial 2

In the second appressoria trial conducted on April 2nd and 3rd, every mutant showed reduced appressoria inhibition at three hours compared to the wild type KJ201, (note: only KJ201, KJB617 and KJB74 have 3-hour data). At 24 hours only KJC86 showed higher appressoria inhibition than the wild type KJ201, (note: there is no 24-hour data for KJB74). Germination was similar in that neither KJB617 or KJB74 had higher inhibition than KJ201 at 3 hours and only KJC35 had higher inhibition than KJ201 at 24 hours.



Graph 8: Second appressoria test showing relation between germination and appressoria inhibition at 3 and 24 hours

Appressoria ratios were compared with bacterial treated trials on the Y-axis and the control on the X-axis at both 3 hours and 24 hours. The scatter plot showed that isolates clustered closer to (1,1) than trial 2 with some variance (see graph 8). Analysis of variance tests were run for appressoria ratios, with control and bacterial treatments as separate groups, at both 3 and 24 hours. Both time periods showed significant variance. At 3 hours there was a very low p-value of 2.555E-10 and low F crit – 2.342 compared to an F-value of 15.482. At 24 hours there was a very low p-value of 1.114E-60 and relatively low crit – 1.861 compared to the very high F-value of 109.268 (see table 6).



Graph 9: Appressoria ratios from trial 2

Table 6: Analysis of variance test for trial 2 appressoria ratios.

ANOVA Appressoria Trial 2 at 3 hours

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.58420976	5	1.11684195	15.4816162	2.5551E-10	2.34182753
Within Groups	5.19407145	72	0.07213988			
Total	10.7782812	77				

ANOVA Appressoria Trial 2 at 24 hours

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.32583558	11	0.48416687	109.268008	1.1138E-60	1.86186771
Within Groups	0.5848924	132	0.004431			
Total	5.91072797	143				

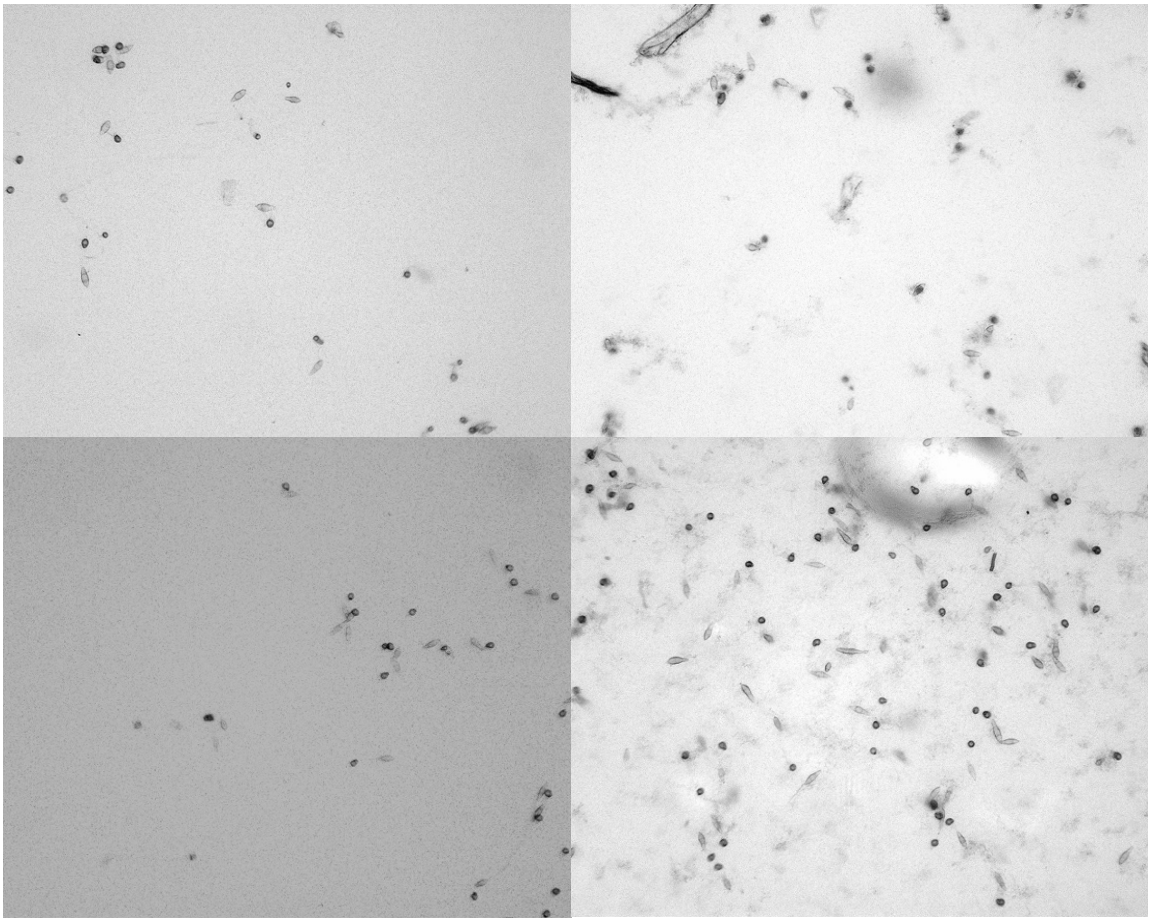


Figure 9: Photographs of appressoria formation after 24hrs. Upper left: KJ201, Upper right: KJ201 + bacterial treatment, Bottom left: KJB67, Bottom right: KJB67 + bacterial treatment

Inverse PCR and molecular studies Sequences are still being processed.

DISCUSSION

Biocontrol is an extremely complicated concept as it includes interactions not just between the control and the pathogen, but the host plant and other microbes as well. As a result, it can be difficult to realistically evaluate the impacts a single organism may have in the context of entire phytobiome. This study demonstrated that induced mutations in *M. oryzae* can indeed lead to ability to overcome and grow through *P.chlororaphis* EA105, but the degree to which the mutants are able to withstand the bacterium may not be enough to eliminate *P.chlororaphis* EA105 as a potential biocontrol.

Direct antagonism test

Antagonism tests did not show a consistent reduction in inhibition. Mycelial growth was greatly reduced in almost every isolate when exposed to *P. chlororaphis* EA105. While some mutants did show a reduction in inhibition, this was not consistent across trials. Analysis of variance tests showed that there was significant variance in between trials, but even wild types Guy 11 and KJ201 were not consistent in their percentage inhibition. As conditions were kept constant across trials, (except for 2.25 as it had only one biological replicate per isolate), it is unclear why the trials showed such variation. One observation that was made is that the plates stacked together in the same foil wrap often showed similar amounts of bacterial growth. A few controls were contaminated with EA105 when wrapped with treatment plates. In future studies it may be advisable to use a dark incubator rather than foil, as it may change the microclimate of the plates.

Appressoria test

Appressoria tests were similarly inconclusive, yet the variance between trials was higher. There was much higher incidence of negative inhibition, even in KJ201 (see graph 6 and 8). This is interesting as the 2014 University of Delaware study showed 90% reduction in appressoria when exposed to *P. chlororaphis* EA105 (Spence et. al. 2014). While the Delaware study used *M. oryzae* 70-15 rather than KJ201 or Guy 11, it is surprising that such a reduction was not seen in this study. This could be explained by differences in calculation or variation between isolates.

Inverse PCR and molecular studies

Sequences are still being processed and will be used in future studies. These sequences may help determine what genes caused the gain of function mutation in the transformed *M. oryzae* isolates. Once these sequences are acquired and analyzed, a good idea may be to generate isolates with a knockout of the gene and expose them to *P. chlororaphis* EA105.

Sources of error

A continual potential source of error was the problem of calculating inhibition. Instead of comparing individual trials, inhibition was calculated using averages. In future studies a better computational strategy might be used to compare between trials. Additionally, it may be advisable to select fewer transformed mutants and increase the number of biological replicates in all assays.

Next steps and questions

While this study is a start, there is still much more to be done to determine how useful this biocontrol may be. First, this study did not test the control in vivo with the host plant. *P. chlororaphis* EA105 has been shown to induce resistance in rice primed with the bacterium (Spence et. al. 2014). It would be interesting to see how the transformed *M. oryzae* isolates differ in infecting primed rice plants. In vivo studies may also help in addressing application questions and how *P. chlororaphis* EA105 might be applied in a way that allows it to interact directly with *M. oryzae*. This leads to the question of life cycles as the two microbes may not be compatible. *P. chlororaphis* EA105 is soil borne, while *M. oryzae* is considered windborne. It is unknown if these two organisms come in contact with each other during infection. *P. chlororaphis* EA105's ability to cause induced resistance in rice still promising nonetheless.

CONCLUSIONS

Mutations in the Rice Blast fungus *M. oryzae* are able to cause increased resistance to *P.chlororaphis* EA105, but this resistance is not consistent and may not be significant enough to discount *P. chlororaphis'* fungistatic ability. Direct antagonism still caused inhibition in the majority of mutants, and appressoria formation was also reduced. More work must still be done to test the biocontrol against mutants in-vivo as well and address dissemination questions. If induced resistance holds up against the transformed *M. oryzae* isolates, *P.chlororaphis* EA105 still has the potential to be an effective biocontrol against the Rice Blast disease.

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